Beer spoilage bacteria and hop resistance in Lactobacillus brevis

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CHAPTER 4

A Plasmid pRH45 of *Lactobacillus brevis* Confers Hop Resistance

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SUMMARY

Lactobacillus brevis ABBC45^{C} was segregated from the original strain ABBC45 after repeating subculturing in the absence of hop compounds. *Lb. brevis* ABBC45^{C} lacks the hop-resistance related plasmid pRH45 that contains *horA* of which the deduced amino acid sequence is 53% identical to LmrA, a lactococcal ATP-binding cassette (ABC) multidrug transporter. *Lb. brevis* ABBC45^{C} is less resistant than ABBC45 to hop compounds and ethidium bromide (EtBr). When pRH45 was re-introduced into *Lb. brevis* ABBC45^{C} by electroporation, the degree of resistance to hop compounds and EtBr was restored to the resistance level of *Lb. brevis* ABBC45. Energized cells of *Lb. brevis* ABBC45^{C} show, in the presence of nigericin, a higher rate of ethidium accumulation than cells of ABBC45. These results indicate that pRH45 confers hop resistance in *Lb. brevis* ABBC45 by excreting hop compounds by the multidrug ABC-type transporter HorA.

INTRODUCTION

The bitter compounds in beer derived from the hop plant are important for the protection of beer from bacterial spoiling (Simpson and Smith, 1992). However, some lactic acid bacteria, especially those belong to *Lactobacillus* spp., exhibit resistance to hop compounds and grow in beer, thereby causing serious problems for the brewing industry. The mechanism(s) of hop resistance of lactobacilli is poorly understood. A biochemical study suggested that unidentified components of the plasma membrane are responsible for the resistance (Simpson and Fernandez, 1994).

In our previous work, we obtained a hop-resistant mutant from *Lb. brevis* ABBC45 (Sami *et al.*, 1997a). This mutant was found to carry a plasmid, pRH45, at a higher copy number than the wild type. Plasmid pRH45 contains *horA*, an open reading frame of 1749 nucleotides (DDBJ accession no. AB005752). HorA has six putative transmembrane domains and an ATP-binding motif (Sami *et al.*, 1997a). The deduced amino acid sequence of HorA shows significant similarity with the bacterial multidrug transporter LmrA (van Veen *et al.*, 1996) and the mammalian multidrug transporter MDR1 (Chen *et al.*, 1986). LmrA has been identified as an ATP-binding cassette (ABC) transporter, which confers resistance of *Lactococcus lactis* to various lipophilic toxic compounds, including ethidium bromide (Bolhuis *et al.*, 1995; van Veen *et al.*, 1996). Almost all lactobacilli isolated as beer-spoilage strains were found to possess a *horA*-like gene (Sami *et al.*, 1997b). However, direct evidence for the involvement of plasmid pRH45 in hop resistance of *Lb. brevis* has not yet been provided. Since elucidation of the mechanism(s) of hop resistance is of crucial importance for the brewing industry, we investigated the contribution of pRH45 in this hop resistance. In this study we obtained a segregant strain ABBC45^{C}, which had spontaneously lost the plasmid pRH45 and which allowed us to demonstrate that *horA* on pRH45 is responsible for hop resistance.

MATERIALS AND METHODS

Bacterial culture

Lactobacillus brevis strains were grown anaerobically at 30ºC in MRS broth (Merck, Darmstadt, Germany, initial pH adjusted to 5.5 with HCl). Anaerobic conditions were generated by AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). Cells were stored in MRS broth containing 20% glycerol at –80ºC.

Segregation of ABBC45^{C}

The wild-type *Lb. brevis* strain, ABBC45, was repeatedly subcultured by inoculating 10^5 cells in 5 ml of MRS broth every 2 to 3 days. After 15 subcultures, single colonies were isolated and plasmid DNAs were purified by the method of

Anderson and McKay (1983). Plasmid profiles of the isolates and ABBC45 were investigated by 0.75% agarose gel electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.01 M EDTA, pH 8.0). A polymerase chain reaction (PCR) was performed using the total DNA extracted from *Lb. brevis* ABBC45^C as a template and two specific primer sets based on the *ori* and the *horA* sequences of pRH45. A Southern blot analysis was also done for the plasmid DNAs of *Lb. brevis* ABBC45^C, using *horA*-specific DNA as a probe.

Re-introduction of pRH45

Re-introduction of pRH45 into *Lb. brevis* ABBC45^C was done by cotransformation by electroporation with a generalized plasmid of lactic acid bacteria, pGK13 (Kok *et al.*, 1984), containing a chloramphenicol-resistant gene. The competent cells were prepared by the method of Sakamoto (See Chapter 3). Cells of *Lb. brevis* ABBC45^C, grown in 50 ml of MRS containing 1% glycine, were harvested by centrifugation at early exponential phase, washed once with cold 3.5 mM $MgCl_2$ and twice with cold SM (925 mM sucrose, 3.5 mM $MgCl_2$) and finally suspended in 500 μl SM. Plasmid DNAs extracted from *Lb. brevis* ABBC45, containing 1 μg of pRH45, were added to 40 μl of competent cell suspension together with 10 ng of pGK13. Electroporation was done at 200 Ω, 2.0 kV and 0.25 μF by using Gene Pulser (Bio-Rad, Hercules, CA, USA), as described (See Chapter 3). Filtered MRSM medium (960 μl total) (MRS broth containing 0.5 M sucrose and 0.1 M $MgCl_2$) was added, and the cell suspension was incubated at 30ºC for 2 h. Cells were harvested by centrifugation (5,500 × g, 4ºC, 5 min), spread on the MRS agar plate containing 15 μg/ml chloramphenicol and 50 μM hop compounds, and incubated anaerobically at 30ºC for 4 days.

Drug resistance

Exponentially growing cells were diluted with sterile deionized water to a concentration of 10^6 cells/ml. These cell suspensions (5 μl) were spotted on MRS agar plates containing various concentrations of hop compounds or EtBr, and the minimum inhibitory concentrations (MICs) were determined.

Ethidium acculmulation

A washed cell suspension of *Lb. brevis* ABBC45^C or ABBC45 in HEPES (50 mM potassium-HEPES supplemented with 3 mM $MgSO_4$, pH 7.5) with an A_{600} of 0.7 was incubated with 10 mM EtBr after the preincubation of cells with 10 mM L-arginine and 4 μM nigericin at 30ºC for 10 min. L-arginine was added to generate ATP by the arginine deiminase pathway (Cunnin *et al.*, 1986) and nigericin for dissipating the transmembrane pH-gradient to prevent the action of pmf-driven transporters. Fluorescence was measured with an F-2000 fluorometer (Hitachi, Tokyo, Japan) for 20 min, using excitation and emission wavelengths of 500 and

580 nm, respectively.

RESULTS

Lb. brevis ABBC45^C lacks both pRH45 and *horA*

A comparison of the plasmid profiles of *Lb. brevis* ABBC45 and of the segregant ABBC45^C obtained after repeated subcultering of ABBC45 in the absence of hop compounds shows that ABBC45^C has lost pRH45, while the other plasmids remained unchanged (Fig. 1). With the specific primer sets for pRH45 and *horA* no PCR products were recognized in the total DNA of *Lb. brevis* ABBC45^C (data not shown). Also with the *horA*-specific DNA probe no discrete band could be detected by a Southern blot analysis with the plasmid DNAs of *Lb. brevis* ABBC45^C (data not shown).

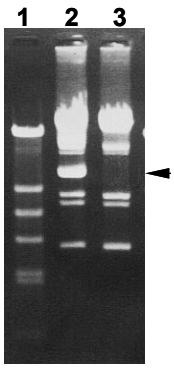

Figure 1. Plasmid profiles of *Lb. brevis* ABBC45.
Plasmids extracted from wild type *Lb. brevis* ABBC45 (lane 2) and ABBC45^C (lane 3), were subjected to agarose gel electrophoresis. Lane 1 contains molecular weight standards (λ DNA digested with *Hind* III). The position of pRH45 is indicated by an arrow at the right side of the figure.

Re-introduction of pRH45

Genetic engineering of lactic acid bacteria is not always possible. Attempts to introduce a marker gene in pRH45 were unsuccessful, possibly due to the instability of several DNA fragments of pRH45 in *E. coli* (Sami *et al.*, 1997a). Cotransformation of *Lb. brevis* ABBC45^C with pGK13 and pRH45 resulted in seven colonies. Examination of the plasmid profiles of these colonies revealed that four colonies had been successfully transformed with both pRH45 and pGK13 (ABBC45^C[pRH45, pGK13]; Fig. 2, lane 7, 8, 11, 12), while the other three colonies contained only pGK13 but not pRH45 (Fig. 2, lane 9, 10, 13). Transformation of *Lb. brevis* ABBC45^C was also done with pGK13 alone (ABBC45^C[pGK13]; Fig. 2, lane 5, 6). Although the copy number of pGK13 in the transformants was small, significant higher resistance to chloramphenicol was realized. Of the four transformants of *Lb. brevis* ABBC45^C[pRH45, pGK13], only one had retained the original plasmid profile of ABBC45^C and pRH45 and pGK13 (Fig. 2, lane 11). This transformant was resistant to hop compounds up to 600 μM while the other transformants were only resistant hop compounds up to 300 μM (data not shown). This hop resistant transformant was used in the following experiments.

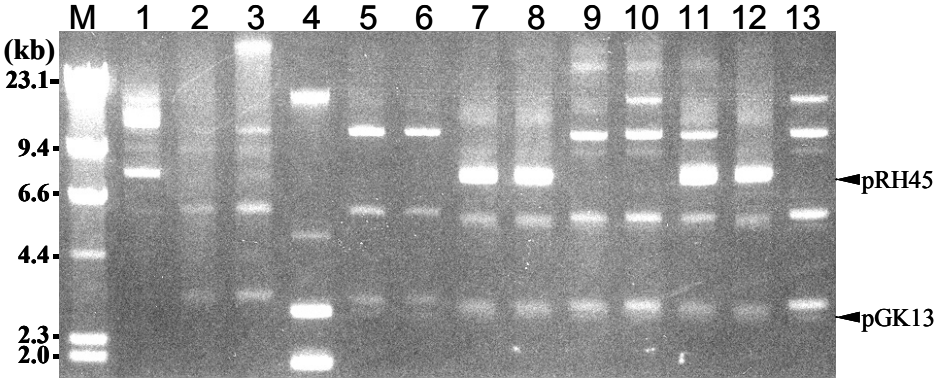

Figure 2. The plasmid profiles of wild type *Lb. brevis* ABBC45, pRH45-free segregant ABBC45^C and transformants of *Lb. brevis* ABBC45^C. Plasmids extracted from ABBC45 (lane 1), the pRH45-free segregant ABBC45^C (lanes 2, 3; different amounts of DNA from the same sample were applied on the gel), ABBC45^C transformed with pGK13 (lanes 5, 6; different amounts of DNA from the same sample were applied on the gel), and seven transformants of ABBC45^C electroporated with pGK13 and pRH45 (lanes 7-13) were subjected to agarose gel electrophoresis. Lane 4 contains pGK13 extracted from the host strain of *E. coli*. The bands around 2.0, 3.0, and 5.0 kb in lane 4 correspond to the closed circular DNA, the open circular DNA, and the linear DNA of pGK13, respectively. The positions of pRH45 and the open circular DNA of pGK13 are indicated by arrows on the right. M: molecular weight standards (λ DNA digested with *Hind* III) and their molecular weight (kb) are indicated on the left.

Drug resistance

Upon loss of pRH45 the MIC to hop compounds of *Lb. brevis* ABBC45 decreased by a factor of 2 and this MIC was completely restored upon the re-introduction of pRH45 (Table 1). These results indicate that pRH45 contributes to resistance of *Lb. brevis* ABBC45 to hop compounds. Similar results were obtained for the resistance to EtBr. These differences in resistance were reproducibly observed in many experiments.

Table 1. Drug resistance of *Lb. brevis* ABBC45

Strains	MIC[a]	
	Hop compounds (μM)	EtBr (μg/ml)
ABBC45 (wild type)	200	30
ABBC45^C	100	15
ABBC45^C[pRH45, pGK13]	200	30
ABBC45^C[pGK13]	100	15

[a]Minimum inhibitory concentrations were determined from cell growth on MRS agar plates containing various concentrations of hop compounds or ethidium bromide. MICs of hop compounds are expressed as iso-α-acids concentrations (Simpson, 1993).

Accumulation of Ethidium

Ethidium readily diffuses across the cell membrane and enters the cytoplasm. Upon intercalation with DNA or RNA its fluorescence increases approximately 10-fold (Le Pecq and Paoletti, 1967). The amount of the intracellular ethidium can therefore be followed fluorometrically. The rate of ethidium accumulation was significantly faster for cells of *Lb. brevis* ABBC45^{C} than for cells of ABBC45 (Fig. 3).

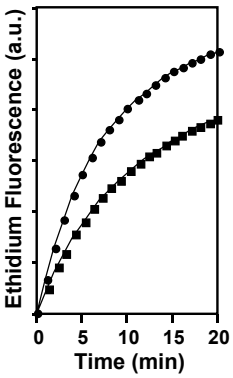

Figure 3. Accumulation of ethidium. The ethidium fluorescence development of cells of a pRH45-free segregant *Lb. brevis* ABBC45^{C} (●) and the wild-type ABBC45 (■) was shown. Cells were preincubated with 10 mM L-arginine and 4 μM nigericin in HEPES (50 mM HEPES, 25 mM K_2SO_4, 5 mM $MgSO_4$, pH 7.5) for 10 min. At zero time the assay was started by the addition of 10 μM ethidium bromide to the cell suspension. The fluorescence (arbitrary units, a.u.) was measured for 20 min by using the excitation wavelength of 500 nm and the emission wavelength of 580 nm.

DISCUSSION

The resistance to hop compounds and EtBr of *Lb. brevis* ABBC45 decreased when pRH45 was lost and completely recovered upon re-introduction of pRH45. In previous studies was found that the copy number of pRH45 increased with the resistance to EtBr and novobiocin when *Lb. brevis* ABBC45 was acclimatized to higher concentrations of hop compounds (Sami *et al.*, 1997a). The excellent correlation of the level of resistance to hop compounds and other drugs with the copy number of pRH45 indicates a crucial role of pRH45 in conferring multidrug resistance.

Ethidium is a substrate of many bacterial multidrug resistant transporters, including BmrB of *Bacillus subtilis* (Neyfakh *et al.*, 1991), QacA of *Staphylococcus aureus* (Tennet *et al.*, 1989), and LmrP (Bolhuis *et al.*, 1995) and LmrA (van Veen *et al.*, 1996) of *Lactococcus lactis*. The rate of ethidium accumulation in an MDR containing bacterium is determined by the rates of diffusion into and the pumping out of the cell (Bolhuis *et al.*, 1994). *Lb. brevis* ABBC45^{C} was found to accumulate ethidium faster than ABBC45 in the presence of nigericin. In these experiments nigericin was used to collapse the transmembrane pH-gradient in order to inhibit proton-motive-force dependent transporters. These results suggest therefore that the activity of the ATP-driven

extrusion system for ethidium is lower in the pRH45-free segregant *Lb. brevis* ABBC45^{C} than in the wild type ABBC45. Among the four transformants of ABBC45^{C}[pRH45, pGK13] only the one, which contained pRH45 and pGK13 and had retained the original plasmid profile of ABBC45^{C}, showed the wild strain ABBC45 level of hop resistance. It is concluded that the multidrug ABC-transporter HorA, encoded by *horA* on pRH45, is responsible for the increased resistance to hop compounds and ethidum.

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